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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The link between obesity and breast cancer development has been postulated but the molecular mechanisms involved are unclear. Leptin, the product of the <i>OB</i> (obesity) gene is a cytokine controlling body fat mass and appetite. The abundance of leptin is greater in females than in males and is regulated by steroid hormones and growth factors, such as estradiol, insulin and insulin-like growth factors. The levels of these factors are elevated in individuals with upper body obesity. This type of obesity correlates with increased breast cancer risk in post-menopausal women. In addition to its role as a regulator of appetite and metabolism, leptin has been shown to act as a chemoattractant and mitogen in several cell systems. We hypothesized that in obese women, locally elevated levels of estrogens and insulin might increase the synthesis of leptin by adipocytes and/or epithelial cells, in effect leading to increased proliferation and/or migration in primary breast tumor. Our data indicate that several breast cancer cell lines express the long and short form of the leptin receptor (Ob-R). Treatment with 100 ng/ml of leptin increased the proliferation of MCF-7 and MDA-MB-435 cells by 30-40%. In MCF-7 cells, the action of leptin was associated with the activation of the ERK1/2 and STAT3 signaling pathways. Estradiol treatment decreased the expression of the Ob-R long form and inhibited leptin growth effects, suggesting the crosstalk between these the estrogen and leptin systems. The additional experiments indicated that estradiol and insulin-like growth factor I treatment did not induce leptin secretion in MCF-7 cells.			
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INTRODUCTION

Obesity in the United States has reached the alarming rate of ~60% and is considered a second, after smoking, major killer. The link between obesity and breast cancer development has been postulated but the molecular mechanisms involved are not clear (1).

Leptin, a 16 kDa protein product of the *OB* (obesity) gene is a cytokine reported to be secreted mainly from adipocytes and has been shown to control body fat mass and food intake by providing information to the central nervous system (2). The abundance of leptin is greater in females than in males and is regulated by steroid hormones and growth factors, such as estradiol, insulin and insulin-like growth factor I (3-7). The levels of these substances are elevated in individuals with upper body obesity. This type of obesity correlates with increased breast cancer risk in post-menopausal women (1). In addition to its role as a regulator of appetite and metabolism, leptin can be involved in other processes, such as hematopoiesis, reproduction, and immunity (2,7). Recently, it has been demonstrated that leptin can act as a mitogen, chemoattractant, and angiogenic factor in different cell models (8-12). New data documented that human breast cancer cell lines and breast tumors may express leptin and leptin receptor (Ob-R) (8,9,13). In addition, leptin has been shown to induce DNA synthesis in MCF-7 and T47D breast cancer cell lines (8,9). We hypothesized that in obese women, locally elevated levels of estrogens and insulin might increase the synthesis of leptin in adipocytes and/or epithelial cells, in effect leading to increased proliferation and/or migration in primary breast tumor.

TECHNICAL REPORT

During this reporting performance period (Year 1) we performed several experiments originally proposed in SOW.

1. Effects of leptin on the growth of breast cancer cells.

Leptin has been shown to induce DNA synthesis in several cell lines (8,9,12). Here we tested whether leptin can stimulate cell proliferation of breast cancer cell lines. The results indicated that leptin at a concentration 100 ng/ml moderately stimulates the growth of estrogen receptor (ER)-positive MCF-7 cells and ER-negative MDA-MB-435 cells (Tab. 1). Lower doses (1 and 10 ng/ml) as well as higher dose of 1000 ng/ml were less effective (data not shown).

Next, we probed the effect of estradiol (E2) on leptin-induced growth in MCF-7 cells. Interestingly, we found that the addition of 10 uM E2 inhibited leptin-dependent proliferation (Tab. 2). Our preliminary data suggest that E2 downregulates the expression of Ob-R (data not shown).

Day	Cell Number ($\times 10^4$)					
	0		2		4	
Leptin	-	+	-	+	-	+
MCF-7 cells	22±3	22±3	28±2	48±4	33±5	51±3
MDA-MB-435 cells	26±4	26±4	41±1	58±6	38±1	54±5

Tab. 1. Effects of leptin on the growth of breast cancer cells. To analyze the growth in the presence of leptin, the cells were plated in 6-well plates at a concentration of $1.5-2.0 \times 10^5$ cells/plate in DMEM:F12 (1:1) containing 5% calf serum. The following day (day 0), the cells at approximately 50% confluence were shifted to serum-free medium (SFM) containing 100 ng/ml leptin or were left untreated (control of basal proliferation in SFM). Cell number was determined at days 0, 2, and 4. The results are average from 3 experiments +/- SD.

	MCF-7 cells + 100 ng/ml leptin				
Day	0		2		4
E2	-	-	+	-	+
Cell Number ($\times 10^4$)	24±3	45±5	30±4	48±5	34±2

Tab. 2. Effects of E2 on leptin-induced growth in MCF-7 cells. The cells were stimulated with 100 ng/ml leptin for 2 and 4 days in the presence or absence of 10 uM E2. Cell number was determined by direct counting. The results are average from 3 experiments +/- SD.

2. Effects of leptin on breast cancer cell migration.

Leptin has been shown to induce invasion or migration in some cell systems (11). We investigated whether leptin can act as a chemoattractant for breast cancer cells. Using Boyden chambers we tested migration of two ER-positive cell lines, MCF-7 and T47D, and two ER-negative cell lines, MDA-MB-231 and MDA-MB-435 (Tab. 3). The results suggested that leptin has negligible effect on the migration of breast cancer cells and regardless of the ER and differentiation status.

Cell Line	Induction of Migration (%)
MCF-7	+ 10±5
T47D	+ 7 ±2
MDA-MB-231	- 11 ±4
MDA-MB-435	+ 15±7

Tab. 3. Effects of leptin on breast cancer cell migration. The effects of leptin were tested on breast cancer cell lines characterized by different degree of differentiation (best MCF-7>T47D>MDA231>MDA453 least). The values represent % decrease/increase relative to untreated cells and are average from 3 experiments +/- SD. The migration of cells was tested using two-well chambers divided with an 8 um porous membrane. 20,000 of cells synchronized for 24 h in SFM and suspended in 200 ul of SFM were plated into the upper chamber. The lower chamber contained 500 ul of either SFM (control of basal migration) or SFM + 100 ng/ml leptin. The cells were allowed to migrate for 24 h. Then, the cells from the upper chamber were removed and the cells that traversed the membrane and attached to its underside were fixed and stained. The number of migrating cells was determined by direct counting under the microscope. The results are average from 3 experiments +/- SD.

3. Expression of leptin receptors in breast cancer cell lines.

At least five different isoforms of the Ob-R have been predicted to exist (14,15). The most ubiquitous are the long and short forms of Ob-R. The long Ob-R has a 302 aa cytoplasmic domain and a 841 aa extracellular domain. The short form Ob-R consists of a small fragment of the cytoplasmic domain (32-40 aa) and a full extracellular domain. Alternative splicing generates other forms that differ mostly in the length of the cytoplasmic domain (14). In addition, a presence of a soluble Ob-R has been described (16). The soluble Ob-R is generated by proteolytic cleavage of the extracellular domain and acts as leptin binding protein regulating leptin availability (6,16). The stimulation of the intracellular signal transmission by leptin requires the ~ 45-65 kDa cytoplasmic domain. The phosphorylation of Tyrosine 985 and 1138 has been shown critical in the activation of STAT3 and ERK1/2 signaling pathways (15).

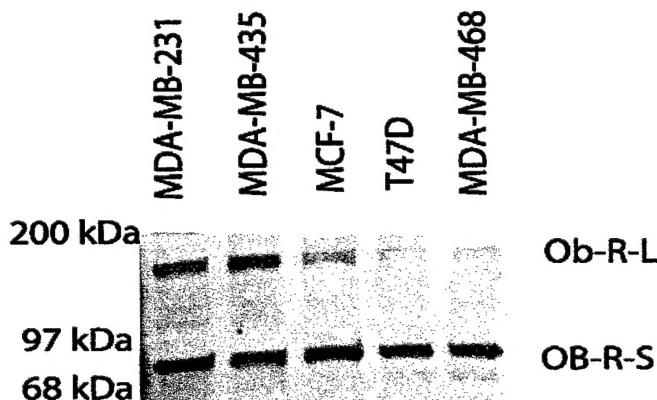


Fig. 1. The expression of Ob-R in different breast cancer cell lines. 70% cultures of cells were lysed using buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM CaCl₂, 100 mM NaF, 0.2 mM Na₃VO₄, 1% PMSF, 2 ug/ml aprotinin. The expression of Ob-R was analyzed in 50 ug of cell lysate by Western blotting with specific anti-Ob-R antibodies (Linco Research). Ob-R long (Ob-L) and short (Ob-S) isoforms are indicated.

4. Leptin signaling in MCF-7 cells.

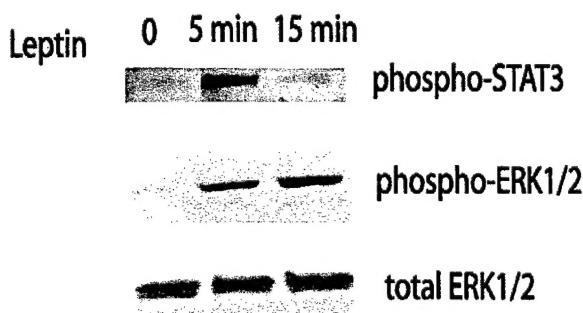
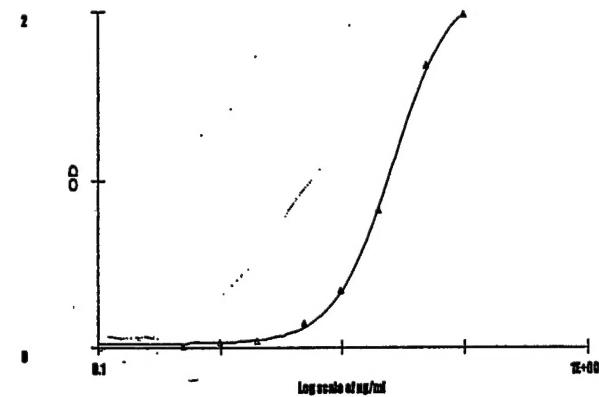


Fig. 2. Leptin signaling in MCF-7 cells. 70% cultures were shifted to SFM for 8 h and then stimulated with 100 ng/ml of leptin for 5 and 15 min. The cells were lysed as described in Fig. 1. The expression of active (phospho) and total ERK1/2 and phospho-STAT3 was analyzed in 50 ug of cell lysates by Western blotting with specific antibodies: anti-active ERK1/2 (Cell Signaling), anti-total ERK1/2 (Cell Signaling), anti-phospho-STAT3 (Santa Cruz).

5. Leptin secretion in E2 and IGF-I stimulated MCF-7 cells.

We tested by ELISA immunoassay whether E2 or insulin-like growth factor I can induce leptin secretion in MCF-7 cells. The measurement of leptin levels in MCF-7 conditioned medium indicated that the mitogens were not able to stimulate leptin production (Fig. 3).



STANDARD	Std. Value 0.5 ng/ml	Well	OD	Mean	Std Dev	CV	Calc. Value
STD01		A1	0.003	0.012	0.021	176.3	<<<<
		A2	0.003				<<<<
		A3	0.003				<<<<
STD02	1 ng/ml	A4	0.048	0.038	0.018	49.94	2.873
		A5	0.047				<<<<
STD03	2 ng/ml	A6	0.044	0.045	2.07e-4	1.539	1.775
		A7	0.045				1.830
STD04	5 ng/ml	A8	0.145	0.140	0.005	2.333	5.311
		A9	0.152				5.301
STD05	10 ng/ml	A10	0.286	0.347	0.026	7.959	10.50
		A11	0.277				9.704
STD06	20 ng/ml	B1	0.708	0.823	0.046	0.588	18.95
		B2	0.855				28.35
STD07	50 ng/ml	B3	1.788	1.814	0.031	1.048	52.88
		B4	1.602				49.88
STD08	100 ng/ml	B5	1.932	1.932	0.014	0.710	82.97
		B6	2.002				100.5

UNKNOWN	Mean	Std Dev	CV	Well	Value	OD	DILFactor
E2 72	—			C9	<<<<	-0.004	1.000
				C10	<<<<	-0.002	
E2 96	—			D1	<<<<	0.916	1.000
				D2	<<<<	0.002	
IGF72	—			C11	<<<<	-0.005	1.000
				C12	<<<<	-0.004	
IGF96	—			D3	<<<<	-0.004	1.000
				D4	<<<<	-0.002	

Fig. 3. ELISA immunoassay for detection of leptin. The assay was used according to the instructions of the manufacturer (Linco Research). Original standard curve with 0.5-100 ng/ml leptin is shown. The samples (UNKNOWNs) were obtained from the conditioned medium of MCF-7 cells treated for 72 or 96 h with E2 or insulin-like growth factor I. The conditioned medium was collected from 90 % confluent cultures of cells grown in 100 mm plates. The medium was concentrated to 0.5 ml final volume and 50 ul were tested by ELISA.

Key Research Accomplishments:

- Demonstrated that leptin can function as a mitogen in breast cancer cells;
- Documented that several breast cancer cell lines express leptin receptors Ob-R-L and Ob-R-S;
- Demonstrated that leptin stimulates ERK1/2 and STAT3 signaling pathways in MCF-7 cells;
- Demonstrated that estradiol inhibits proliferative effects of leptin in MCF-7 cells.

Reportable Outcomes:

1. Manuscripts, abstracts and scientific presentations:

Manuscripts: None

Abstracts:

1. Sauter ER, Garofalo C, Hewitt J, Surmacz E. Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA; University of Missouri-Columbia, Columbia, MO.

Leptin expression in nipple aspirate fluid (NAF) is influenced by BMI and menopausal status. Breast Cancer Symposium, San Antonio, 2002, submitted

2. Patents and licenses: None

3. Degrees: N/A

4. Development of biologic reagents: None

5. Databases: None

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2003-2006, DOD Breast Cancer Research Program "Association of Leptin (the obesity gene) with Breast Cancer", \$100,000/yr, 5% Collaborator (PI E. Sauter), pending

7. Employment applied for: None

Conclusions

Leptin (obesity protein) may play a role in breast cancer development by stimulating the growth of breast cancer cells. Estradiol appears to downregulate the effects of leptin, thus, leptin might be an important mitogen for ER-negative breast cancer cells.

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